

### AMENDMENTS TO THE SPECIFICATION

Please delete paragraphs [0012], [0014], [0015], [0020], and [0022].

Please replace paragraph [0013] with the following amended paragraph:

[0013] ~~Figure 2 depicts~~ Figures 1A-C depict the quantitation of inflammation and immunostaining positive cells. Panels A and B show the counts of inflammatory foci and the scores of inflammation that were quantitated from immunized-only, control-infused and rFasL-infused rat LSSC sections. Panel C shows the quantitation of ED1, OX19, OX42, W3/13, W3/25 immunostaining positive cells in control-infused and rFasL-infused rat LSSC sections. (\*p<0.05)

Please replace paragraph [0016] with the following amended paragraph:

[0016] ~~Figure 5 depicts~~ Figures 2A-H depict Annexin V-FITC/PI staining and flow cytometry and ~~shows~~ show that rFasL dose-dependently induced apoptosis in MBP-activated T blasts. Because virtually all gamma-irradiated thymocytes were positive in PI staining after two days in culture (panel A), most PI-negative cells in co-culture were the bigger T blasts (panels B & C). With the doses of rFasL from 0 to 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml, T blasts double negative for Annexin V-FITC and PI staining among total cells decreased significantly from 42.9% to 20.5%, 10.5%, 6.1% and 3.7% respectively. (Panels D to H) The results were typical for three separate experiments.

Please replace paragraph [0017] with the following amended paragraph:

[0017] ~~Figure 6 demonstrates~~ Figures 3A-B demonstrate that in vitro treatment of MBP-specific T line cells with 200 ng/ml rFasL for 16 hours abrogated the encephalitogenicity in T line cells.  $2 \times 10^6$  non-treated T blasts were intravenously transferred into each of four naive Lewis rats, which invariably developed 3<sup>0</sup> EAE, (panel A) and reduced over 30 grams in body weight during EAE. (Panel B) In contrast, cells from duplicate culture but treated with rFasL

were collected and transferred in the same way as non-treated cells but could not transfer EAE at all.

Please replace paragraph [0018] with the following amended paragraph:

**[0018]** Figure ~~[[7]]~~ 4 demonstrates that rFasL treatment dose-dependently induced cell death in activated macrophages. Peritoneal inflammatory macrophages were activated in vitro with 100 U/ml IFN-gamma for 24 hours and then triggered with 200 ng/ml of LPS. rFasL, either alone or with the anti-FLAG antibody that cross-links rFasL, was added at the same time as LPS. MTT assay was performed 16 hours later. The results were typical for three separate experiments.

Please replace paragraph [0019] with the following amended paragraph:

**[0019]** Figure ~~[[8]]~~ 5 demonstrates rFasL dose-dependently potentiated the inhibition of T cell proliferation by rat CSF. Zero to 50% (V/V) rat CSF was included from the start of T cell proliferation experiment. rFasL was added 24 hrs later. [<sup>3</sup>H]-thymidine was added another 24 hours later, and cells were harvested further 16 hours later. The results were representative for three separate experiments.

Please replace paragraph [0021] with the following amended paragraph:

**[0021]** ~~Figure 10 demonstrates~~ Figures 6A-B demonstrate that the prevention of EAE by rFasL infusion was not due to the suppression of systemic immune response to MBP. Panel A compares the MBP-induced DTH responses in immunized only, control-infused, and rFasL-infused rats on 12 dpi, which do not show any significant differences among these three groups of animals. Panel B compares the MBP-induced T cell proliferation between the control infused, and the rFasL infused rats on both 10 dpi and 12 dpi, which again does not show any significant differences. (p>0.05)

Please replace paragraph [0023] with the following amended paragraph:

**[0023]** Figure [[12]] 7 depicts the amino acid sequence of a full length human Fas ligand (GenBank accession number P48023) SEQ ID NO: 1.

Please replace paragraph [0134] with the following amended paragraph:

**[0134]** Since inflammation is most severe in the LSSC in this EAE model (Simmons R. D. et al. (1992) Autoimmunity 14:17-21; Matsuda M. et al. (1994) Autoimmunity. 19:15-22), the degree of inflammation in LSSC was compared between four control-infused rats (all with 3<sup>0</sup> EAE) and four rats infused with 350 ng of rFasL (three were EAE-free, and one with 1<sup>0</sup> EAE). The tissues were all obtained on 12 dpi. HE staining (~~FIG. 1A~~) shows that control-infused rats at the peak EAE stage developed severe inflammation in the LSSC, most significantly in the meningeal and perivascular areas, but many inflammatory cells also infiltrated into the parenchyma of the spinal cord. In contrast, minimal inflammation was observed in LSSC of rFasL-infused rats (~~FIG. 1B~~). In the rat that was infused with 350 ng of rFasL and developed mild EAE, the degree of inflammation in the LSSC was also found to be much milder. Previous studies have suggested that T lymphocytes and macrophages represent most of the inflammatory cells in EAE (Raine C S. (1984) Lab Invest. 50:608-635). To compare the presence of these two cell populations in LSSC sections between the control infusion group and the rFasL infusion group, immunostaining was performed with multiple markers. ED1 staining is a marker for macrophages, but also weakly stains the granulocytes. OX19 (anti-rat CD5) and W3/13 (anti-rat CD43) are markers mainly for the T cells, but also stain some B cells and polymorphonuclear cells respectively. OX42 is a marker for microglia and infiltrated macrophages, and W3/25 is a marker for CD4<sup>+</sup> cells. All of these markers showed significant decreases following 350 ng rFasL infusion (~~typical ED1 and OX19 staining are shown in FIGS. 1C, D, E, F~~). Thus, intrathecal rFasL infusion reduced the degree of inflammation, the numbers of ED1<sup>+</sup> cells and

OX19+ cells in both the meningeal, perivascular areas and the spinal cord parenchyma, when examined by micrographs of HE staining, ED1, and OX19 immunostaining of control-infused EAE rat LSSC sections or of corresponding staining of rFasL-infused EAE-prevented rat LSSC sections.

Please replace paragraph [0135] with the following amended paragraph:

[0135] To quantitate the degrees of inflammation in LSSC, two pathological indices were compared, i.e. the count of inflammatory foci and the score of inflammation, in three groups of animals: non-infused EAE rats (n=3, EAE 3<sup>0</sup>), control-infused EAE rats (n=4, EAE 3<sup>0</sup>), 350 ng rFasL-infused rats (n=4, three were EAE-free and one with 1<0>EAE). (Fig. 2 Figs. 1A-C) RFasL infusion significantly reduced the count of inflammatory foci by 80% and the score of inflammation by 83%, but control infusion had no significant effect on both parameters. This suggests that while control infusion did not affect LSSC inflammation, rFasL infusion greatly inhibited spinal cord inflammation in this EAE model. The cells that were positive in ED1, OX42, OX19, W3/13, and W3/25 immunostaining were quantitated and it was found that rFasL infusion decreased positively stained cells by 91%, 73%, 89%, 91% and 94% respectively. (Fig. 2C Fig. 1C) The less reduction in OX42 positive cells could be due to the inclusion of some OX42+ resident microglia (Spanaus K. S. et al (1998) Eur J ImmunoL 28:4398-4408). Taken together, the data indicate that 350 ng rFasL infusion greatly reduced the infiltration of T cells and macrophages in the rat LSSC.

Please replace paragraph [0136] with the following amended paragraph:

[0136] Studies were performed to determine 1) whether infiltrated T cells and macrophages in the EAE spinal cord express Fas receptors, 2) whether neurons, astrocytes or oligodendrocytes in the normal spinal cord express Fas receptors, and 3) how the pattern of Fas+ cells was changed after rFasL infusion. Double immunolabeling clearly showed that Fas

receptors were expressed on both OX19+ cells and ED1+ cells, suggesting that these inflammatory cells could be the targets for rFasL (~~FIG. 3A, B~~). Anti-GFAP, Rip and SMI-32 monoclonal antibodies were specific markers for astrocytes, oligodendrocytes and neurons respectively. Double labeling of Fas on GFAP, Rip, and SMI-32 immunostaining positive cells in the normal rat spinal cord was observed, suggesting Fas receptors were constitutively expressed on astrocytes, oligodendrocytes, and neurons in the rat spinal cord. (~~FIGS. 3C, D, E~~) When Fas immunostaining was compared between control-infused and rFasL-infused rat LSSC sections, rFasL infusion was found to dramatically decrease the incidence of Fas+ cells in LSSC. (~~FIGS. 3F, G~~) The remaining Fas+ cells after rFasL infusion exhibited the same pattern of Fas immunostaining as in the normal rat LSSC sections (~~data not shown~~), suggesting that most of them were Fas+ spinal cord neural cells, which were not affected by the rFasL infusion. Thus, Fas receptors are highly expressed in ED1+ and OX19+ cells in EAE rat LSSC, and are constitutively expressed on astrocytes, neurons and oligodendrocytes in normal rat LSSC. RFasL infusion greatly reduced Fas+ cells, and the remaining Fas+ cells showed same pattern as in normal rat LSSC, as demonstrated by double immunostaining with Fas antibody and OX19, ED1, Rip, GFAP, and SMI-32 antibodies respectively, and comparing the anti-Fas immunostaining in a control-infused EAE rat LSSC section and a rFasL-infused EAE-prevented rat LSSC section.

Please replace paragraph [0138] with the following amended paragraph:

[0138] First, T cells lines from the draining lymph nodes of MBP-immunized Lewis rats were established. After the second round of in vitro MBP stimulation,  $1 \times 10^6$  T blasts were sufficient to transfer  $3^0$  EAE (complete hind limb paralysis) in the recipient naive rats. RFasL was tested on these T cells. As shown in FIG. 4A, non-treated Non-treated T line cells were almost confluent in culture, and they were much bigger than co-cultured gamma-irradiated thymocytes. After 16 hrs of treatment with 200 ng/ml rFasL, very few T blasts were still alive. Thus, in vitro rFasL treatment induced morphologically apoptotic death in MBP-specific T line

cells. T line cells were in the second round of MBP stimulation with gamma-irradiated thymocytes as APCs. After 24 hours of MBP stimulation, control cells were not treated, and the duplicate culture was treated with 200 ng/ml rFasL. Sixteen hours later, non-treated T blasts were almost confluent and were much bigger in sizes compared with co-cultured gamma-irradiated thymocytes. In the dish that was treated with rFasL, most T blasts were dead or dying, and very few remained normal morphology. Many dying T blasts show typical morphologic apoptotic changes. (FIG. 4B) The great susceptibility of these T blasts to rFasL was further shown in Annexin V-FITC/propidium iodide (PI) staining analyzed by flow cytometry. ~~(Fig. 5~~ Figs. 2A-H) Because virtually all gamma-irradiated thymocytes were positive in PI staining after two days in culture (Fig. ~~[[5A]]~~ 2A), most PI-negative cells in co-culture were the bigger T blasts (Figs. ~~5B, 5C~~ 2A, 2C). With the doses of rFasL from 0 to 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml, T blasts double negative for Annexin V-FITC and PI staining among total cells decreased significantly from 42.9% to 20.5%, 10.5%, 6.1% and 3.7% respectively. ~~(Figs. 5D to 5H~~ 2D to 2H) Over 90% of T blasts were killed by 200 ng/ml rFasL treatment within 16 hrs.

Please replace paragraph [0139] with the following amended paragraph:

**[0139]** Next, the changes in encephalitogenicity of T line cells after rFasL treatment were examined in an adoptive transfer EAE model. Although  $1 \times 10^6$  T blasts were sufficient to transfer  $3^0$  EAE,  $2 \times 10^6$  T blasts were transferred into each of the four naive rats, and the cells from duplicate culture but treated with 200 ng/ml rFasL for 16 hours were transferred into each of other four naive rats. (Controlled by same cell collection and transferring procedures) As shown in ~~Fig. 6~~ Figs. 3A-B, four rats transferred with non-treated T blasts all developed  $3^0$  EAE, and the average body weight decrease during the EAE course was over 30 grams. In contrast, none of those four rats transferred with rFasL treated cells developed any symptom of EAE, or had any decrease in their body weights. These data show that rFasL treatment is able to completely abrogate the encephalitogenicity of MBP-specific T line cells within 16 hours.

Please replace paragraph [0140] with the following amended paragraph:

**[0140]** In order to determine whether activated macrophages were also susceptible to rFasL-mediated killing, peritoneal inflammatory macrophages were first activated with 100 U/ml IFN-gamma for 24 hours, and then triggered with lipopolysaccharide (LPS). A dose-dependent effect in killing macrophages was observed with rFasL treatment for 16 hrs. (Fig. [[7]] 4) Up to 45% of macrophages were killed by 200 ng/ml rFasL treatment. When an anti-FLAG(R) antibody (1.5 µg/ml) which crosslinks rFasL by the FLAG(R) tail was added, enhanced killing effects were observed, and over 70% of activated macrophages were eliminated with 200 ng/ml rFasL. These results suggest that while activated macrophages are not as sensitive to rFasL as activated T line cells, majority of activated macrophages are susceptible to FasL-induced cell death, depending on the format of FasL that is administered.

Please replace paragraph [0141] with the following amended paragraph:

**[0141]** The effects of rat CSF alone or together with different doses of rFasL on MBP-induced proliferation in MBP-specific T line cells was also examined. (Fig. [[8]] 5) Different percentages (V/V) of CSF, or HBSS (as controls) were included from the beginning of T cell proliferation experiment. While 10%-50% HBSS had no significant effect on T cell proliferation, the inclusion of 10%-50% CSF dose-dependently inhibited T cell proliferation. The inclusion of 50% CSF inhibited 76% of T cell proliferation. This inhibitory effect was further enhanced dose-dependently by rFasL treatment. With 50% CSF in culture, any tested dose of rFasL reduced T cell proliferation by over 90%. These data suggest that while CSF has a profound immunosuppressive function, exogenous FasL greatly potentiate this suppression.

Please replace paragraph [0143] with the following amended paragraph:

**[0143]** To study the in vivo mechanism of EAE suppression by rFasL infusion, the

TUNEL staining patterns were compared between control-infused and rFasL-infused animals. When control-infused rats (n=3) reached their EAE peak, TUNEL+ cells appeared mostly in the parenchyma of LSSC (~~FIG. 4A~~), but were rare in the meningeal and perivascular areas (~~FIGS. 9A, C~~). In rFasL-infused rats (n=3) that had no EAE symptoms on 12 dpi, the TUNEL+ cells were less common in both the parenchyma and meningeal/perivascular areas. In a LSSC section from a control-infused, 3<sup>0</sup> EAE rat, TUNEL+ cells appeared mostly in the parenchyma of LSSC, but were rare in the inflammatory meningeal and perivascular areas. In a LSSC section from a rFasL-infused, EAE-free rat, the TUNEL+ cells were less common in both the parenchyma and meningeal/perivascular areas, where inflammation is minimal. However, in another section from a rFasL-infused, 1<sup>0</sup> EAE (complete tail paralyzed) rat, the TUNEL+ cells were greatly increased in the meningeal and perivascular areas of LSSC, where mild inflammation is present. (FIG. 9B)

The same situation was observed when TUNEL staining was performed on LSSC sections obtained on 10 dpi from rFasL-infused and EAE free rats (n=3). This was expected since the inflammation was minimal in LSCC of rFasL-infused and EAE free rats at all time points examined, i.e. on 9 dpi, 10 dpi, 12 dpi and 15 dpi. (~~Some data not shown~~) This suggested that the total number of inflammatory cells entering the CNS after rFasL infusion was much less than that in controls. In the development of EAE, only small numbers of activated antigen-specific T cells first cross the blood-tissue (in this case the blood-brain) barrier and enter the CNS perivascular areas. With the pro-inflammatory feedback from these cells, a much larger second-wave infiltration of both T cells and macrophages leads to the severe CNS inflammation and the initiation of EAE disease (Wekerle H, et al. (1994) Ann Neurol. 36:S47-53).

Please replace paragraph [0144] with the following amended paragraph:

[0144] To determine whether the suppression of acute EAE after intrathecal rFasL infusion might also contributed by the suppression of the systemic immune response to MBP, the MBP-induced DTH response on 12 dpi, and MBP-induced T cell proliferation on both 10 dpi and



12 dpi between control-infused and 350 ng rFasL-infused, EAE-free animals were compared. As shown in Fig. [[10]] 6, neither the DTH responses nor the T cell proliferation to MBP differed significantly between the two groups. These results exclude the possibility that systemic immune deviation or tolerance to MBP played a role in suppressing the acute EAE after rFasL infusion.

Please replace paragraph [0147] with the following amended paragraph:

**[0147]** As mentioned above, neurons, astrocytes, and oligodendrocytes in the rat spinal cord constitutively were found to express Fas receptors. This raised the possibility that rFasL infusion might cause cytotoxicity in normal neural cells, although the patterns of Fas<sup>+</sup> cells in LSSC were normal after rFasL infusion. Immunostaining with anti-GFAP, Rip and SMI-32 antibodies on 700 ng rFasL-infused and normal rat LSSC sections was compared. No changes were observed in either the densities or the morphology of these neural cells (~~FIGS. 11A-D, neuronal staining not shown~~). Luxol fast blue staining on 700 ng rFasL-infused LSSC sections, demonstrated no white matter areas with myelin loss (~~FIG. 11E~~). Toluidine blue staining was also performed on semi-thin LSSC sections from 700 ng rFasL infused rats. It showed that the myelin sheaths were intact and the myelin thickness was normal (~~FIG. 11F~~). In conclusion, no toxic effects on the neural cells or the myelin structure in LSSC was observed after infusion of 700 ng rFasL. Thus, rFasL infusion did not damage astrocytes, oligodendrocytes or the myelin structure in rat LSSC as demonstrated by micrographs showing anti-GFAP and Rip staining in normal non-immunized rat LSSC sections and in corresponding staining in rFasL-infused rat LSSC sections, which show that astrocytes and oligodendrocytes are present in same densities and staining patterns. Luxol fast blue staining of rFasL-infused LSSC sections did not show any area with myelin loss. Toluidine blue staining on semi-thin LSSC sections showed intact myelin structure and normal myelin thickness.